

APPLICATION FOR  
UNITED STATES LETTERS PATENT  
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UNITED STATES PATENT AND TRADEMARK OFFICE  
(Case No. PFI-010CN/71369.362)

Title:  
**P<sub>2x7</sub> RECEPTOR-DEFICIENT NON-HUMAN ANIMALS  
AND USES THEREOF**

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**P<sub>2x7</sub> RECEPTOR-DEFICIENT NON-HUMAN ANIMALS AND  
USES THEREOF**

5                                    CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of Application No. 09/875,318, filed on June 6, 2001, which claims the benefit of U.S. Provisional Application No. 60/209,559, filed June 6, 2000.

10                                   FIELD OF THE INVENTION

The present invention relates to non-human, genetically modified mammals and genetically modified animal cells having a disruption in a P<sub>2x7</sub> receptor gene locus that prevents or reduces endogenous P<sub>2x7</sub> receptor function.

15                                   BACKGROUND OF THE INVENTION

Cell surface ATP receptors can be divided into the metabotropic receptor family (P<sub>2Y</sub>/P<sub>2U</sub>) and the ionotropic receptor family (or the P<sub>2x</sub> receptor family).  
20    Metabotropic receptor family members are G-protein coupled receptors and ionotropic receptor family members are ligand-gated channels. There are eleven metabotropic receptor family members and seven ionotropic receptor family members, P<sub>2x1</sub>R to P<sub>2x7</sub>R.

The P<sub>2x7</sub> receptor (P<sub>2x7</sub>R), like other members of the P<sub>2x</sub> receptor family, is an  
25    ATP-gated ion channel (Surprenant *et al.* (1996) *Science* **272**:735-738; Rassendren *et al.* (1997) *J. Biol. Chem.* **272**:5482-5486; Michel *et al.* (1998) *Br. J. Pharmacol.* **125**:1194-1201). The P<sub>2x7</sub>R, however, demonstrates attributes that clearly distinguish it from other members of the family. For example, the P<sub>2x7</sub>R requires levels of ATP in excess of 1 mM to achieve activation whereas other P<sub>2x</sub> receptors activate at ATP  
30    concentrations ≤100 μM (Greenberg *et al.* (1988) *J. Biol. Chem.* **263**:10337-10343; Steinberg *et al.* (1987) *J. Biol. Chem.* **262**:8884-8888): the higher concentration requirement reflects, in part, the preference of the P<sub>2x7</sub>R for ATP<sup>4-</sup> as its ligand and the relatively low abundance of this species in media containing physiological concentrations of divalent cations (*e.g.*, Ca<sup>2+</sup> and Mg<sup>2+</sup>). An additional unique feature

5 of the P<sub>2x7</sub>R is found in its conductance properties. All P<sub>2x</sub> receptors demonstrate non-selective channel-like properties following ligation, but the channels formed by the P<sub>2x7</sub>R rapidly transform to “pores” that allow passage of solutes as large as 900 daltons (Steinberg *et al.* (1987) *J. Biol. Chem.* **262**:8884-8888; Virginio *et al.* (1999) *J. Physiol.* **519**:335-346). Molecular details of this transformation remain to be  
10 described, but domain swapping and deletion experiments have suggested that the carboxy terminal domain of the P<sub>2x7</sub>R participates in pore complex formation; the carboxy terminal domain is significantly longer than the comparable domains in the other P<sub>2x</sub> receptors (North (1996) *Current Opin. Cell Biol.* **8**:474-483). Possibly as a consequence of this pore-like activity, continuous ligation of the P<sub>2x7</sub> receptor for  
15 times greater than 15 minutes can lead to cell death (Di Virgilio (1995) *Immunol. Today* **16**:524-528; Murgia *et al.* (1992) *Biochem. J.* **288**:897-901; Ferrari *et al.* (1999) *FEBS Let.* **447**:71-75).

The P<sub>2x7</sub>R displays a restricted cellular distribution, being observed primarily in cells of hematopoietic origin including monocytes and macrophages and some  
20 lymphocyte populations (Di Virgilio (1995) *Immunol. Today* **16**:524-528; Collo *et al.* (1997) *Neuropharmacol.* **36**:1277-1283). The receptor also has been reported to exist on microglial cells (Sanz *et al.* (2000) *J. Immunol.* **164**:4893-4898), some cancer cells (Wiley *et al.* (1989) *Blood* **73**:1316-1323), sperm (Foresta *et al.* (1996) *Am J. Physiol.* **270**:C1709-C1714), and dendritic cells (Mutini *et al.* (1999) *J. Immunol.* **163**:1958-  
25 1965). The P<sub>2x7</sub>R has been reported to participate in a diverse list of cellular activities including lymphocyte proliferation (Baricordi *et al.* (1999) *J. Biol. Chem.* **274**:33206-33208), fertilization (Foresta *et al.* (1996) *Am J. Physiol.* **270**:C1709-C1714), giant cell formation (Chiozzi *et al.* (1997) *J. Cell Biol.* **138**:697-706), cell death (Murgia *et al.* (1992) *Biochem. J.* **288**:897-901; Ferrari *et al.* (1999) *FEBS Let.* **447**:71-75),  
30 killing of invading mycobacteria (Lammas *et al.* (1997) *Immunity* **7**:433-444), and IL-1 posttranslational processing (Hogquist *et al.* (1991) *Proc. Natl. Acad. Sci. (USA)* **88**:8485-8489; Perregaux *et al.* (1994) *J. Biol. Chem.* **269**:15195-15203). Further, ligation of the P<sub>2x7</sub>R has been associated with activation of phospholipase D and activation of some forms of NF- $\kappa$ B (Humphreys *et al.* (1996) *J. Immunol.* **157**:5627-  
35 5637; Ferrari *et al.* (1997) *J. Cell Biol.* **139**:1635-1643).

One of the most intriguing activities attributed to the P<sub>2x7</sub>R is its ability to induce posttranslational processing of proIL-1 (Sanz *et al.* (2000) *J. Immunol.*

5      164:4893-4898; Hogquist *et al.* (1991) *Proc. Natl. Acad. Sci. (USA)* **88**:8485-8489;  
Perregaux *et al.* (1994) *J. Biol. Chem.* **269**:15195-15203). Interleukin (IL)-1 is a  
multipotential inflammatory mediator produced in abundance by activated monocytes  
and macrophages. The administration of IL-1 to animals has been shown to initiate an  
inflammatory response, produce fever, and promote tissue degradation. Further,  
10      elevated levels of IL-1 have been detected in patients suffering from a number of  
chronic disorders, including rheumatoid arthritis, Alzheimer's disease, and acute  
myelocytic leukemia (McNiff *et al.* (1995) *Cytokine* **7**:209; Gray *et al.* (1986)  
*J. Immunol.* **137**:3644; Lomedico *et al.* (1984) *Nature* **312**:458).

When IL-1 is released from cells, it binds to receptors on target cells and  
15      elicits complex signaling cascades leading to the upregulation of gene products that  
contribute to an inflammatory state including matrix metalloproteinases,  
cyclooxygenase-2, IL-6, and cellular adhesion molecules (Flannery *et al.* (1999)  
*Matrix Biol.* **18**:225-237; Guzn *et al.* (1998) *J. Biol. Chem.* **273**:28670-28676; Allen  
*et al.* (2000) *J. Exp. Med.* **191**:859-869; Bevilacqua *et al.* (1989) *Science* **243**:1160-  
20      1164). Two distinct gene products, IL-1 $\alpha$  and IL-1 $\beta$ , contribute to IL-1 biological  
activity. The amino acid sequences of IL-1 $\alpha$  and IL-1 $\beta$  are only 25% identical yet  
these two polypeptides bind to the same receptors on target cells (Slack *et al.* (1993)  
*J. Biol. Chem.* **268**:2513-2524). Human IL-1 $\alpha$  and IL-1 $\beta$  are both initially produced as  
31 kDa procytokines containing amino terminal extensions that are subsequently  
25      removed by proteolysis. In the case of proIL-1 $\alpha$ , the propolypeptide and the 17 kDa  
cleavage product display equivalent signaling activity indicating that proteolytic  
cleavage is not necessary to generate a receptor-competent ligand. In contrast, proIL-  
1 $\beta$  does not bind to the signaling IL-1 receptor (Mosley *et al.* (1987) *J. Biol. Chem.*  
**262**:2941-2944), and cleavage by caspase-1 is necessary to generate the mature  
30      17 kDa signaling-competent form of this cytokine (Cerretti *et al.* (1992) *Science*  
**256**:97-100; Thornberry *et al.* (1992) *Nature* **356**:768-774).

The two forms of IL-1 share another very unusual attribute, both proIL-1 $\alpha$  and  
proIL-1 $\beta$  are synthesized without a signal sequence (March *et al.* (1985) *Nature*  
**315**:641-647), the peptide epitope required to direct nascent polypeptides to the  
35      endoplasmic reticulum (Walter *et al.* (1994) *Ann. Rev. Cell Biol.* **10**:87-119). As a  
result, newly synthesized proIL-1 $\alpha$  and proIL-1 $\beta$  accumulate within the cytoplasmic  
compartment of producing cells rather than being sequestered to the secretory

5 apparatus. Caspase-1 also is produced as a cytosol-localized proenzyme, the 45 kDa  
propolypeptide must be proteolytically processed to generate the 20 kDa and 10 kDa  
subunits which constitute the mature active protease (Thornberry *et al.* (1992) *Nature*  
**356**:768-774; Miller *et al.* (1993) *J. Biol. Chem.* **268**:18062-18069; Ayala *et al.*  
(1994) *J. Immunol.* **153**:2592-2599). In activated monocytes and macrophages,  
10 therefore, proIL-1 $\beta$  and procaspase-1 co-exist within the cytoplasm. Mechanisms that  
control activation of procaspase-1, and in turn cleavage of proIL-1 $\beta$ , are not well  
understood. Recent studies, however, have provided evidence that proteolytic  
processing IL-1 $\beta$  and release of the mature cytokine product extracellularly do not  
proceed constitutively. Rather, the post-translational processing of proIL-1 requires  
15 that lipopolysaccharide- (LPS)-activated monocytes and/or macrophages encounter an  
external stimulus that promotes activation of procaspase-1, cleavage of proIL-1 $\beta$ , and  
release of the 17 kDa cytokine (Miller *et al.* (1995) *J. Immunol.* **154**:1331-1338;  
Laliberte *et al.* (1999) *J. Biol. Chem.* **274**:36944-36951; Sanz *et al.* (2000) *J.*  
*Immunol.* **164**:4893-4898). Stimuli that function *in vitro* to promote IL-1  
20 posttranslational processing by LPS-activated monocytes and/or macrophages include  
ATP, nigericin, cytolytic T-cells, bacterial toxins and hypotonic stress (Bhakdi *et al.*  
(1990) *J. Clin. Invest.* **85**:7988-7992; Hogquist *et al.* (1991) *Proc. Natl. Acad. Sci.*  
(USA) **88**:8485-8489; Perregaux *et al.* (1992) *J. Immunol.* **149**:1294-1303; Perregaux  
*et al.* (1994) *J. Biol. Chem.* **269**:15195-15203; Perregaux *et al.* (1996) *J. Immunol.*  
25 **157**:57-64; Walev *et al.* (1995) *EMBO J.* **14**:1607-1614). This requirement for  
secretion stimulus is not restricted to cells in culture; mouse peritoneal macrophages  
produce proIL-1 $\beta$  in response to intraperitoneal (i.p.) injection of LPS, but release  
little cytokine extracellularly (Griffiths *et al.* (1995) *J. Immunol.* **154**:2821-2828).  
Subsequent i.p. injection of ATP, however, stimulates generation of large quantities of  
30 extracellular mature IL-1 $\beta$  (Griffiths *et al.* (1995) *J. Immunol.* **154**:2821-2828).

#### SUMMARY OF THE INVENTION

35 The present invention provides non-human, genetically- modified mammals  
and animal cells containing a disrupted P<sub>2x7</sub>R gene that prevents or reduces  
endogenous P<sub>2x7</sub>R function.

5           In one aspect, the invention provides a genetically-modified, non-human mammal, wherein the genome of the mammal comprises a genetic modification to a copy of the  $P_{2x7}R$  gene that results in disrupted  $P_{2x7}R$  gene function from the modified gene. In one embodiment, the mammal is heterozygous for the modified  $P_{2x7}R$  gene. In another embodiment, the mammal is homozygous for the modified  $P_{2x7}R$  gene.

10           In preferred embodiments, the mammal is a rodent, such as a mouse. In some embodiments, the mouse is homozygous for the modified  $P_{2x7}R$  gene and exhibits reduced ATP-stimulated intracellular translocation of macromolecules. In other embodiments, the mouse is homozygous for the modified  $P_{2x7}R$  gene and exhibits reduced ATP-stimulated interleukin- (IL)-1 $\alpha$ , IL-1 $\beta$ , or IL-18 posttranslational  
15           processing or reduced ATP-stimulated IL-6 production.

          In some embodiments, the  $P_{2x7}R$  gene is disrupted by homologous recombination, and in other embodiments, the  $P_{2x7}R$  gene is disrupted by the insertion of a gene trapping vector.

          The invention provides, in another aspect, a method for producing a  
20           genetically-modified mouse comprising a disrupted  $P_{2x7}R$  gene. In this method, a nucleic acid molecule is introduced into a mouse embryonic stem cell, wherein the nucleic acid molecule inserts into the  $P_{2x7}R$  gene, thereby disrupting the gene. In one embodiment of the method of the invention, the nucleic acid molecule disrupts the  $P_{2x7}R$  gene by homologous recombination. In another embodiment, the nucleic acid  
25           molecule disrupts the  $P_{2x7}R$  gene by the insertion of a gene trapping vector. The mouse embryonic stem cell containing the introduced nucleic acid is then introduced into a mouse embryo, which is then transplanted into a pseudopregnant mouse. The embryo containing the introduced mouse embryonic stem cell is allowed to develop to term, after which a genetically-modified mouse is identified whose genome comprises  
30           a disruption of a  $P_{2x7}R$  gene.

          In another aspect, the invention provides an isolated nucleic acid molecule comprising a  $P_{2x7}R$  gene targeting construct. Upon introduction of the targeting construct into a cell, the construct recombines with a  $P_{2x7}R$  gene in the cellular genome, thereby inserting itself at the  $P_{2x7}R$  gene locus and disrupting  $P_{2x7}R$  gene  
35           function in the cell.

          The invention provides, in yet another aspect, a genetically-modified animal cell comprising a genetic modification in at least one copy of  $P_{2x7}R$  gene in the

5 genome of the cell, the genetic modification resulting in disrupted function in the  
modified copy of P<sub>2x7</sub>R gene. In some embodiments, the cell is a mammalian cell. In  
a preferred embodiment, the cell is an embryonic stem cell. In some embodiments,  
the cell is derived from a genetically-modified, non-human mammal or non-human  
mammalian embryo. In some embodiments, the cell is heterozygous for the modified  
10 P<sub>2x7</sub>R gene, and in other embodiments the cell is homozygous for the modified P<sub>2x7</sub>R  
gene.

Various terms used herein are defined as stated below.

By a non-human mammal or an animal cell that is “genetically-modified” is  
meant such a mammal or cell having a modification in a gene of interest which was  
15 introduced into the non-human mammal or cell, or a progenitor mammal or cell, by  
genetic engineering. The non-human mammal or cell is heterozygous or homozygous  
for the modified gene. In a genetically-modified, non-human mammal, it is preferred  
that all somatic cells and germline cells contain the modified gene.

This modification disrupts P<sub>2x7</sub>R gene function and features the insertion of a  
20 foreign nucleic acid sequence into the P<sub>2x7</sub>R gene locus, either alone or in combination  
with a deletion of the endogenous P<sub>2x7</sub>R gene sequence, and the modification can  
occur within any region of the P<sub>2x7</sub>R gene, *e.g.*, in an enhancer, promoter, regulator  
region, noncoding region, coding region, intron, or exon.

By a nucleic acid sequence that is “exogenous” or “foreign” to a P<sub>2x7</sub>R gene is  
25 meant a sequence that is non-naturally occurring in the P<sub>2x7</sub>R gene. Portions of the  
exogenous sequence may be either “homologous” or “heterologous” to P<sub>2x7</sub>R gene.  
By “homologous” is meant a sequence that is related to a P<sub>2x7</sub>R gene to a degree  
sufficient to allow *in vivo* hybridization. By contrast, a “heterologous” sequence is  
unrelated to P<sub>2x7</sub>R gene and does not hybridize *in vivo* with a P<sub>2x7</sub>R gene.

30 By “disrupted P<sub>2x7</sub>R gene function” is meant a decrease in the P<sub>2x7</sub>R  
polypeptide activity encoded by the modified P<sub>2x7</sub>R gene. When the genetic  
modification in a non-human mammal or animal cell effectively eliminates all wild  
type copies of the P<sub>2x7</sub>R gene (*e.g.*, the non-human mammal or animal cell is  
homozygous for the P<sub>2x7</sub>R gene disruption or the only wild type copy of the P<sub>2x7</sub>R  
35 gene originally present in the genome of the non-human mammal or animal cell is  
now disrupted), then the P<sub>2x7</sub>R gene disruption results in a statistically significant  
reduction in the P<sub>2x7</sub>R polypeptide activity, as compared to the wild type, non-human

5 mammal or animal cell of the same strain or type. This reduction in P<sub>2x7</sub>R polypeptide activity results from reduced expression of the P<sub>2x7</sub>R gene (*i.e.*, reduced P<sub>2x7</sub>R mRNA levels produce reduced levels of P<sub>2x7</sub>R polypeptide) and/or because the modified P<sub>2x7</sub>R gene encodes a mutated polypeptide with reduced function compared to a wild type P<sub>2x7</sub>R polypeptide. Preferably, the activity of the P<sub>2x7</sub>R polypeptide is reduced to  
10 about 50% or less of wild type levels, more preferably, to about 25% or less, and, even more preferably, to about 10% or less of wild type levels. Most preferably, the P<sub>2x7</sub>R polypeptide activity is nondetectable in the genetically modified, non-human mammal or animal cell (*i.e.*, the P<sub>2x7</sub>R gene disruption results in a null mutation).

By “pseudopregnant” mouse is meant a female mouse with hormone levels  
15 comparable to a pregnant mouse and sufficient to maintain a pregnancy. A pseudopregnant mouse is prepared by mating a female mouse in natural estrus with a vasectomized or genetically sterile male mouse.

By “reduced” is meant a statistically significant decrease (*i.e.*,  $p < 0.1$ ).

By “modulates” is meant a statistically significant increase or decrease in  
20 level.

By “P<sub>2x7</sub>R polypeptide activity” or “P<sub>2x7</sub>R-like activity” is meant an increased ATP-stimulated cell permeability and an increased accumulation of a macromolecule, *e.g.*, a dye macromolecule such as YoPro Yellow, an increase in ATP-stimulated IL-1 $\alpha$ , IL-1 $\beta$ , or IL-18 posttranslational processing in activated inflammatory cells  
25 (*e.g.*, activated by lipopolysaccharide treatment or cytokine treatment, such as tumor necrosis factor- $\alpha$ ), an increase in ATP-stimulated IL-6 production in inflammation-induced cells, or lymphoproliferation.

The term “animal cell” is meant to include a cell in an immortalized cell line, in a primary cell preparation, or otherwise derived from an animal. Preferably, the  
30 animal is a mammal such as a human, mouse, or rat.



BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other aspects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

Figure 1 is a diagrammatic representation of a homologous recombination event occurring between an endogenous locus (the murine  $P_{2x7}R$  genomic locus) and a targeting vector. The top line represents the  $P_{2x7}R$  genomic locus. The middle line depicts the targeting vector with a *KpnI-BamHI* (B) fragment (the neomycin resistance gene) inserted into an exon directly after the Arg<sup>505</sup> codon. The targeted locus after homologous recombination is shown at the bottom; filled gray boxes indicate exons and labeled boxes indicate the PGK-TK and PGK-Neo selection cassettes.

Homologous recombination of the targeting vector with the endogenous  $P_{2x7}$  gene disrupts the carboxy (COOH) terminal coding region of the  $P_{2x7}$  gene. Relevant restriction sites are abbreviated as follows, B, *BamHI*; TV, *EcoRV*; K, *KpnI*; S, *SalI*;

Figure 2A is a representation of a Southern blot of the  $P_{2x7}R$  genomic region in  $P_{2x7}R^{+/+}$ ,  $P_{2x7}R^{+/-}$ , and  $P_{2x7}R^{-/-}$  mice in which DNA from pups derived from a heterozygous mating. DNA was digested with *EcoRV* and hybridized with the probe indicated by a dotted line in Figure 1. This probe detects a 16 Kb band from the endogenous locus and a 10 Kb band from the targeted locus;

Figure 2B is a representation of a Northern blot showing the absence of  $P_{2x7}R$  RNA expression in  $P_{2x7}R^{-/-}$  mice. RNA from cultured bone marrow derived mast cells from  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  mice was analyzed by Northern blot analysis for  $P_{2x7}R$  expression using a cDNA probe specific for the  $P_{2x7}R$  mRNA. Equal loading of the lanes and the integrity of the mRNA obtained from the  $P_{2x7}R^{-/-}$  cells were confirmed by analysis of the Northern blot with an actin specific probe;

5        Figure 3 is a representation of a Western blot showing the absence of  $P_{2x7}R$  protein in the  $P_{2x7}R^{-/-}$  macrophages. Lysates from peritoneal macrophages of  $P_{2x7}R^{-/-}$  mice were tested for expression of  $P_{2x7}R$  by Western Blot analysis: lane 1,  $P_{2x7}R^{+/+}$  macrophages; lane 2,  $P_{2x7}R^{-/-}$  macrophages; lane 3, MRC-5 Fb (negative control); lane 4,  $P_{2x7}R$  expressing HEK293 (positive control);

10        Figure 4A is a graphic representation of the accumulation of YoPro Yellow in  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  macrophages in response to ATP stimulation. Peritoneal macrophages isolated from  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  mice were treated with 5 mM ATP in the presence of YoPro Yellow and fluorescence was measured using a microtiter plate reader;

15        Figure 4B is a graphic representation of the accumulation of YoPro Yellow in  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  mice in response to saponin. Peritoneal macrophages isolated from  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  mice were treated with 0.0075% saponin in the presence of YoPro Yellow and fluorescence was measured using a microtiter plate reader;

20        Figures 5A and 5B are representations of an autoradiograph which demonstrates stimulus-coupled IL-1 $\beta$  posttranslational processing in  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  macrophages. Peritoneal macrophages isolated from  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  mice were stimulated with lipopolysaccharide (LPS), radiolabeled with  $^{35}S$ -methionine, and stimulated with 5 mM ATP, 20  $\mu$ M nigericin, or no effector, for 30 minutes. The supernatant (Fig. 5B) and macrophages (Fig. 5A) were harvested independently and the samples were immunoprecipitated for IL-1 $\beta$ . Immunoprecipitates were electrophoresed on an SDS-polyacrylamide gel, from which an autoradiograph was developed;

25        Figure 6A is a graphic representation showing the percentage of IL-1 $\beta$  posttranslational processing in  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  macrophages. Peritoneal macrophages isolated from  $P_{2x7}R^{+/+}$  and  $P_{2x7}R^{-/-}$  mice were stimulated with LPS, radiolabeled with  $^{35}S$ -methionine, and stimulated with 5 mM ATP, or 20  $\mu$ M nigericin, or without an effector, for 30 minutes. The supernatant and macrophages were harvested independently and the samples were immunoprecipitated for IL-1 $\beta$ .

5 Immunoprecipitates were run on an SDS-polyacrylamide gel and the quantity of radioactivity associated with an individual IL-1 $\beta$  polypeptide species was determined by scanning dried gels with a phosphoimager. This figure depicts the percentage of the total radiolabeled IL-1 $\beta$  recovered as the 17 kDa IL-1 $\beta$  polypeptide;

10 Figure 6B is a graphic representation showing the percentage of lactate dehydrogenase (LDH) released from the total culture associated LDH in the samples described in Figure 6A;

15 Figure 7 is a graphic representation showing the abolishment of ATP-induced IL-1 $\beta$  release in P<sub>2x7</sub>R<sup>-/-</sup> mice. Mice were either injected i.p. with LPS or left untreated. Two hours later, phosphate buffered saline (10 mM phosphate, 150 mM NaCl) (PBS) or ATP was injected i.p. into LPS-primed mice. Following an additional 30 minute incubation, the peritoneal cavity was lavaged and the amount of IL-1 $\beta$  recovered was measured by ELISA; and

20 Figure 8 is a graphic representation showing ATP-induced IL-6 release in LPS primed mice. Wild-type mice were primed by an injection of LPS (or PBS) at time 0. Two hours later an injection of ATP (or PBS) was administered to promote IL-1 posttranslational processing and the mice were incubated for an additional 4 hours. 25 Peritoneal lavages were collected at several times during the course of the experiment and analyzed for IL-6 by ELISA.

5

DETAILED DESCRIPTION

10 The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, published foreign applications, and references cited herein are hereby incorporated by reference.

15 The present invention provides non-human, genetically modified animals, preferably genetically modified mice, which harbor a disruption in a  $P_{2x7}R$  gene locus that functionally disrupts an endogenous  $P_{2x7}R$  gene locus. The invention encompasses non-human, genetically modified animals which are heterologous ( $P_{2x7}R^{+/+}$ ) or homozygous ( $P_{2x7}R^{-/-}$ ) for  $P_{2x7}R$  gene disruption. Preferably, the germline and somatic cells of the non-human genetically modified animals contain the disruption of the  $P_{2x7}R$  gene locus.

20 The present invention also provides animal cells, including human cells, having a disruption in a  $P_{2x7}R$  gene locus. Preferably, the disruption prevents the expression of the endogenous  $P_{2x7}R$  gene. The invention encompasses embryonic stem cells that are heterozygous or homozygous for  $P_{2x7}R$  gene disruption. Preferably, the embryonic stem cells are mouse embryonic stem cells. In an alternative embodiment, the genetically modified cells are human cells. The present invention also provides cells obtained from a non-human, genetically modified mammal that is heterologous or homozygous for  $P_{2x7}R$  gene disruption. These cells may be in the form of primary cell preparations or immortalized cell lines.

25 The non-human, genetically modified animals contemplated by the present invention generally include any non-human mammals which encode a  $P_{2x7}R$  gene or homolog thereof. Such non-human genetically modified animals may include, for example, genetically modified pigs, genetically modified rats, genetically modified rabbits, genetically modified cattle, genetically modified goats, and other genetically modified mammalian species known in the art, such as chimpanzee. Preferably, the animals are mice and most preferably the animals are non-human primates.

30 Gene targeting, which is a method of using homologous recombination to modify a mammalian genome, can be used to introduce changes into cultured cells. By targeting a gene of interest in embryonic stem (ES) cells, these changes can be introduced into the germlines of laboratory animals to study the effects of the

5 modifications on whole organisms, among other uses. The gene targeting procedure is accomplished by introducing into tissue culture cells a DNA targeting construct that has a segment homologous to a target locus and which also comprises an intended sequence modification (*e.g.*, insertion, deletion, point mutation). The treated cells are then screened for accurate targeting to identify and isolate those which have been properly targeted. A common scheme to disrupt gene function by gene targeting in ES cells is to construct a targeting construct which is designed to undergo a homologous recombination with its chromosomal counterpart in the ES cell genome. The targeting constructs are typically arranged so that they insert additional sequences, such as a positive selection marker, into coding elements of the target gene, thereby functionally disrupting it. Targeting constructs usually are insertion-type ("knock in;" see, *e.g.*, Hanks *et al.* (1995) *Science* **269**:679-682; and Wang *et al.*, (1996) *Nature* **379**:823-825) or replacement-type constructs ("knock out;" Hasty *et al.* (1991) *Mol. Cell. Biol.* **11**:4509).

The non-human, genetically modified animals and cells that have at least one P<sub>2x7</sub>R locus inactivated can be prepared by any of several techniques that are well established in the art including, but not limited to, those cited herein. For example, techniques such as homologous recombination gene trapping, or mutagenesis induced chemically or by irradiation may be used, as described in U.S. Patent No. 5,464,764; U.S. Patent No. 5,859,312; Shastry (1994) *Mol. Cell. Biochem.* **136**:171-182; Shastry, B.S. (1995) *Experientia* **51**:1028-1039; Bronson *et al.* (1994) *J. Biol. Chem.* **264**:27155-27158; Capecchi (1989) *Science* **244**:1288-1292; Smithies *et al.* (1995) *Proc. Natl. Acad. Sci. (USA)* **92**:5266-5272; Gatherer, D. (1993) *Dev. Growth Diff.* **35**:365-370; and Rossant (1991) *Curr. Opin. Genet. Dev.* **1**:236-240. In general, cells that have at least one P<sub>2x7</sub>R locus inactivated may be engineered by:

- 30 (1) constructing a targeting vector comprising a cloning vector and a DNA fragment containing at least one positively selectable marker gene (positive selection marker), flanked by two regions that are homologous to the animal's P<sub>2x7</sub>R gene or genomic locus and which are in the same 5' to 3' orientation to one another (referred to as the regions of homology);
- 35 (2) including in the targeting vector a negatively selectable marker gene (negative selection marker) adjacent to one of the regions of homology. This negatively selectable marker may increase the likelihood of recovering the desired

5 homologous recombination event (deleting a portion of the P<sub>2x7</sub>R gene) but it is not required;

(3) transfecting P<sub>2x7</sub>R wild-type animal cells with the targeting vector of step (2);

10 (4) selecting the transfected cells from step 3 for the marker(s) on the vector; and

(5) screening for cells with at least one P<sub>2x7</sub>R locus inactivated from those cells in step (4) which are found to contain or express said positive selection marker(s), and not express said negative selection marker(s).

15 General principles regarding the construction of targeting constructs are reviewed in Bradley *et al.* (*BioTechnol.* (1992) **10**:534). The configuration of a targeting construct depends upon the specific targeting technique chosen. For example, a targeting construct for single-crossover integration or "hit-and-run" targeting need only have a single homology clamp linked to the targeting region, whereas a double-crossover replacement-type targeting construct requires two  
20 homology clamps, one flanking each side of the replacement region. P<sub>2x7</sub>R targeting constructs of the invention have a polynucleotide sequence comprising: (1) at least one P<sub>2x7</sub>R homology region having a sequence that is substantially identical to or substantially complementary to a sequence present in a host cell endogenous P<sub>2x7</sub>R gene locus; and (2) a targeting region which becomes integrated into a host cell  
25 endogenous P<sub>2x7</sub>R gene locus by homologous recombination between a targeting construct homology region and said endogenous P<sub>2x7</sub>R gene locus sequence. Typically, targeting constructs of the invention are used for functionally disrupting an endogenous P<sub>2x7</sub>R gene and comprise at least two homology regions separated by a nonhomologous sequence which contains an expression cassette encoding a selectable  
30 marker, such as neo (Smith and Berg (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49**:171; Sedivy and Sharp (1989) *Proc. Natl. Acad. Sci. (USA)* **86**:227; Thomas and Capecchi (1987) *Cell* **51**:503).

Other markers that could be used in the targeting construct include hyg, hisD, gpt, ble, hppt,  $\beta$ -galactosidase,  $\beta$ -lactamase, firefly luciferase, or green fluorescent  
35 protein (see, *e.g.*, Capecchi *et al.*, U.S. Patent No. 5,464,764; Capecchi (1989) *Science* **244**:1288-92; Current Protocols in Cytometry, Units 9.5 and 9.6, John Wiley & Sons, New York, NY, 2000).

5 In a preferred embodiment, a targeting construct comprises, in order: (1) a first  
homology clamp having a sequence substantially identical to a sequence within about  
3 kilobases upstream (*i.e.*, in the direction opposite to the translational reading frame  
of the  $P_{2x7}R$  gene exons) of an exon of an endogenous  $P_{2x7}R$  gene; (2) a replacement  
region comprising a positive selection cassette having a promoter driving transcription  
10 of a neo gene; (3) a second homology clamp having a sequence substantially identical  
to a sequence within about 3 kilobases downstream of said exon of said endogenous  
 $P_{2x7}R$  gene; and (4) a negative selection cassette. Such a targeting construct is suitable  
for double-crossover replacement recombination which deletes a portion of the  
endogenous  $P_{2x7}R$  gene locus spanning said exon and replaces it with the replacement  
15 region having the positive selection cassette. If the deleted exon is essential for  
expression of a functional  $P_{2x7}R$  gene product, the resultant exon-depleted allele is  
functionally disrupted and is termed a null allele.

Targeting constructs or portions thereof comprising at least one portion of a  
nucleotide sequence that is substantially homologous to a sequence present in or  
20 flanking an endogenous  $P_{2x7}R$  gene locus integrate by homologous recombination and  
result in a functional disruption in the expression of  $P_{2x7}R$  from the gene locus. If the  
targeting construct is a "hit-and-run" or "in-and-out" type construct (Valancius and  
Smithies (1991) *Mol. Cell. Biol.* 11:1402; Donehower *et al.* (1992) *Nature* 356:215;  
Donehower *et al.* (1991) *J. NIH Res.* 3:59), the  $P_{2x7}R$  targeting region is only  
25 transiently incorporated into the endogenous  $P_{2x7}R$  gene locus and is eliminated from  
the host genome by selection. A  $P_{2x7}R$  targeting region may comprise a sequence that  
is substantially homologous to an endogenous  $P_{2x7}R$  gene sequence and/or may  
comprise a nonhomologous sequence, such as a selectable marker (*e.g.*, neo, tk, gpt).  
The term "targeting construct" does not necessarily indicate that the polynucleotide  
30 comprises a gene which becomes integrated into the host genome; nor does it  
necessarily indicate that the polynucleotide comprises a complete structural gene  
sequence. As used in the art, the term "targeting construct" is synonymous with the  
term "targeting transgene" as used herein. Targeting constructs are generally double-  
stranded DNA molecules, most usually linear.

35 A "homology region" or "homology clamp" is a segment (*i.e.*, a portion) of a  
 $P_{2x7}R$  targeting construct having a sequence that substantially corresponds to, or is  
substantially complementary to, a predetermined endogenous  $P_{2x7}R$  gene sequence,

5 which can include sequences flanking said  $P_{2x7}R$  gene. A homology region is generally at least about 100 nucleotides long, preferably at least about 250 to 500 nucleotides long, typically at least about 1000 nucleotides long or longer. Although there is no demonstrated theoretical minimum length for a homology clamp to mediate homologous recombination, it is believed that homologous recombination efficiency generally increases with the length of the homology clamp. Similarly, the recombination efficiency increases with the degree of sequence homology between a targeting construct homology region and the endogenous target sequence, with optimal recombination efficiency occurring when a homology clamp is isogenic with the endogenous target sequence. The specific regions of homology required in the targeting construct depend upon the way in which a  $P_{2x7}R$  gene locus is disrupted. The regions of homology are generally present in a targeting construct in the same 5' to 3' orientation relative to one another. The terms "homology clamp" and "homology region" are interchangeable as used herein. Endogenous  $P_{2x7}R$  gene sequences that substantially correspond to, or are substantially complementary to, a transgene homology region are referred to herein as "crossover target sequences" or "endogenous target sequences."

Homology regions that are isogenic to the target cells generally enhance the efficiency of gene targeting. The polynucleotide sequences that can be used as homology clamps in a targeting construct can be obtained, *e.g.*, from GenBank database, in literature publications, or by routine cloning and sequencing, *etc.*  $P_{2x7}R$  polynucleotide sequences include, but are not limited to, those polynucleotide sequences found in Genbank Accession No. NM\_0011207, Genbank Accession No. NM\_002562, and Genbank Accession No. X95882. Typically, a  $P_{2x7}R$  gene sequence is used as a basis for producing PCR primers that flank a region that will be used as a homology clamp in a targeting construct. The PCR primers are then used to amplify, by high fidelity PCR amplification (Mattila *et al.* (1991) *Nucl. Acids Res.* 19:4967; Eckert and Kunkel (1991) *PCR Methods Appl.* 1:17; and U.S. Patent No. 4,683,202), a genomic sequence from a genomic clone library or from a preparation of genomic DNA, preferably from the strain of non-human animal that is to be targeted with the targeting construct. The amplified DNA is then used as a homology clamp and/or targeting region. Thus, homology clamps for targeting essentially any  $P_{2x7}R$



5 gene may be readily produced on the basis of nucleotide sequence information available in the art and/or by routine cloning.

A  $P_{2x7}R$  targeting region is a portion of a  $P_{2x7}R$  targeting construct that becomes integrated into an endogenous  $P_{2x7}R$  chromosomal location following homologous recombination between a homology clamp and an endogenous  $P_{2x7}R$  gene sequence. Typically, a  $P_{2x7}R$  targeting region is flanked on each side by a  $P_{2x7}R$  homology clamp, such that a double-crossover recombination between each of the homology clamps, and their corresponding endogenous  $P_{2x7}R$  gene sequences, results in replacement of the portion of the endogenous  $P_{2x7}R$  gene locus by the targeting region; in such double-crossover gene replacement targeting constructs the targeting region can be referred to as a " $P_{2x7}R$  replacement region." However, some targeting constructs may employ only a single  $P_{2x7}R$  homology clamp (*e.g.*, some "hit-and-run"-type vectors; see, Bradley *et al.* (1992) *BioTechnol.* **10**:534). Homology regions and targeting regions are linked together in conventional linear polynucleotide linkage (5' to 3' phosphodiester backbone).

20 A  $P_{2x7}R$  replacement region is a portion of a  $P_{2x7}R$  targeting construct flanked by  $P_{2x7}R$  homology regions. Upon double-crossover homologous recombination between flanking homology regions and their corresponding endogenous  $P_{2x7}R$  gene crossover target sequences, the replacement region is integrated into the host cell chromosome between the endogenous crossover  $P_{2x7}R$  target sequences. Replacement regions can be homologous (*e.g.*, have a sequence similar to the endogenous  $P_{2x7}R$  gene sequence but have a point mutation or a missense mutation), nonhomologous (*e.g.*, a *neo* gene expression cassette), or a combination of homologous and nonhomologous regions.

Generally, targeting constructs of the invention preferably include: (1) a positive selection expression cassette flanked by two homology regions that are substantially identical to host cell endogenous  $P_{2x7}R$  gene sequences; and (2) a distal negative selection expression cassette. However, targeting constructs which include only a positive selection expression cassette can also be used. Typically, a targeting construct contains a positive selection expression cassette which includes a *neo* gene under the control of a promoter, such as the HSV tk promoter or the pgk promoter. More typically, the targeting construct will also contain a negative selection

5 expression cassette which includes an HSV tk gene linked downstream of a HSV tk promoter.

The positive selection expression cassette encodes a selectable marker which affords a means for selecting cells which have integrated targeting transgene sequences spanning the positive selection expression cassette. The negative selection  
10 expression cassette encodes a selectable marker which affords a means for selecting cells which do not have an integrated copy of the negative selection expression cassette. Thus, by a combination positive-negative selection protocol, it is possible to select cells that have undergone homologous replacement recombination and incorporated the marker between the homology regions (*i.e.*, the replacement region)  
15 into a P<sub>2x7</sub>R gene by selecting for the presence of the positive marker and for the absence of the negative marker. Selectable markers typically are also used for hit-and-run targeting constructs and selection schemes (Valancius and Smithies (1991) *Mol. Cell. Biol.* 11:1402).

Positive-negative selection involves the use of two active selection cassettes:  
20 (1) a positive one (*e.g.*, the *neo* gene) that can be stably expressed following either random integration or homologous targeting, and (2) a negative one (*e.g.*, the HSV *tk* gene) that is stably expressed following random integration, but not after integration of the construct following correctly targeted double-crossover homologous recombination. By combining both positive and negative selection steps, host cells  
25 having the correctly targeted homologous recombination between the transgene and the endogenous P<sub>2x7</sub>R gene can be obtained.

Other negative selectable markers (and their agents of selection) include Hprt (hypoxanthine), and diphtheria toxin, ricin toxin, or cytosine deaminase (all selected by 5-fluorocytosine). Further examples of positive-negative selection schemes are  
30 described, for example, in U.S. Patent No. 5,464,764 and in WO 94/06908. It is recognized, however, that one or more negative selectable markers are not required in the targeting vector in order to carry out the present invention.

An expression cassette typically comprises a promoter which is operational in the targeted host cell (*e.g.*, ES cell) linked to a structural sequence that encodes a  
35 protein or polypeptide that confers a selectable phenotype on the targeted host cell, and a polyadenylation signal. A promoter included in an expression cassette may be constitutive, cell type-specific, stage-specific, and/or modulatable (*e.g.*, by hormones

5 such as glucocorticoids; MMTV promoter), but is expressed prior to and/or during  
selection. An expression cassette can optionally include one or more enhancers,  
typically linked upstream of the promoter and within about 3-10 kilobases. However,  
when homologous recombination at the targeted endogenous site(s) places the  
nonhomologous sequence in the proper reading frame downstream of a functional  
10 endogenous promoter, it may be possible for the targeting construct replacement  
region to comprise only a structural sequence encoding the selectable marker, and rely  
upon the endogenous promoter to drive transcription (Doetschman *et al.* (1988) *Proc.*  
*Natl. Acad. Sci. (USA)* **85**:8583). Similarly, an endogenous enhancer located near the  
targeted endogenous site may be relied on to enhance transcription of transgene  
15 sequences in enhancerless transgene constructs. Preferred expression cassettes of the  
invention encode and express a selectable drug resistance marker and/or a HSV  
thymidine kinase enzyme. Suitable drug resistance genes include, for example: gpt  
(xanthine-guanine phosphoribosyltransferase), which can be selected for with  
mycophenolic acid; neo (neomycin phosphotransferase), which can be selected for  
20 with G418 or hygromycin; and dihydrofolate reductase (DHFR), which can be  
selected for with methotrexate (Mulligan and Berg (1981) *Proc. Natl. Acad. Sci.*  
*(USA)* **78**:2072; Southern and Berg (1982) *J. Mol. Appl. Genet.* **1**:327; which are  
incorporated herein by reference).

25 Selection for correctly targeted recombinants generally employ at least positive  
selection, wherein a nonhomologous expression cassette encodes and expresses a  
functional protein (*e.g.*, neo or gpt) that confers a selectable phenotype to targeted  
cells harboring the endogenously integrated expression cassette, so that, by addition of  
a selection agent (*e.g.*, G418 or mycophenolic acid) such targeted cells have a growth  
or survival advantage over cells which do not have an integrated expression cassette.

30 It is preferable that selection for correctly targeted homologous recombinants  
also employ negative selection, so that cells bearing only nonhomologous integration  
of the transgene are selected against. Typically, such negative selection employs an  
expression cassette encoding the herpes simplex virus thymidine kinase gene (HSV  
35 *tk*) positioned in the transgene so that it would integrate only by nonhomologous  
recombination. Such positioning generally is accomplished by linking the HSV *tk*  
expression cassette (or other negative selection cassette) distal to the recombinogenic

5       homology regions so that double-crossover replacement recombination of the  
homology regions transfers the positive selection expression cassette to a  
chromosomal location but does not transfer the HSV tk gene (or other negative  
selection cassette) to a chromosomal location. A nucleoside analog, gancyclovir,  
which is preferentially toxic to cells expressing HSV *tk*, can be used as the negative  
10       selection agent, as it selects for cells which do not have an integrated HSV tk  
expression cassette. FIAU may also be used as a selective agent to select for cells  
lacking HSV *tk*.

Any of a wide variety of cloning vectors may be used to construct the  
P<sub>2x7</sub>R-targeting constructs of the present invention. Examples of such cloning vectors  
15       include, but are not limited to, pBR322 and pBR322-based vectors (Sekiguchi (1983)  
*Gene* 21:267), pMB9, pBR325, pKH47, pBR328, pHc79, phage Charon 28  
(Boehringer Mannheim Biochemicals, Indianapolis, IN), pKB11, pKSV-10 (P-L  
Biochemicals), and oligonucleotide (dg)-tailed pBR322, pBluescript or similar  
plasmids (Stratagene, La Jolla, CA), pK19 or related plasmids (New England Biolabs,  
20       Beverly, MA), the pUC series of plasmids (New England Biolabs, Beverly, MA), the  
pGEM series of plasmids (Promega, Madison, WI), and the like.

The specific host employed for propagating the targeting constructs of the  
present invention is not critical, but the host will preferably have a functional *hsd*  
modification system. Examples of such hosts include *E. coli* K12 RR1 (Bolivar *et al.*  
25       (1977) *Gene* 2:95); *E. coli* K12 HB101 (ATCC No. 33694); *E. coli* MM21 (ATCC  
No. 336780); and *E. coli* DH1 (ATCC No. 33849). Similarly, alternative  
vector/cloning systems could be employed such as targeting vectors which grow in  
*E. coli* or *Saccharomyces cerevisiae*, or both, or plasmid vectors which grow in *B.*  
*subtilis* (Ure *et al.* (1983) *Meth. Enzymol.* 101: Part C).

30       Targeting constructs propagated in such hosts as *E. coli* are isolated using  
standard molecular biology methods, or may be synthesized as oligonucleotides.  
Targeting transgenes can be transferred to host cells by any suitable technique,  
including microinjection, electroporation, lipofection, biolistics, calcium phosphate  
precipitation, and viral-based vectors, among others. Other methods used to transform  
35       mammalian cells include the use of Polybrene, protoplast fusion, and others (see,  
generally, Sambrook *et al.* Molecular Cloning: A Laboratory Manual, 2d ed., 1989,

5 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The preferred method is electroporation.

It is preferable to use a transfection technique with linearized transgenes containing only modified target gene sequence(s) and without vector sequences. The modified gene site is such that a homologous recombinant between the exogenous  
10 targeting construct and the endogenous DNA target sequence can be identified by using carefully chosen primers and PCR or by Southern blot analysis, followed by analysis to detect if PCR products or Southern blot bands specific to the desired targeted event are present (Erlich *et al.* (1991) *Science* **252**:1643).

Several studies have already used PCR to successfully identify the desired  
15 transfected cell lines (Zimmer and Gruss (1989) *Nature* **338**:150; Mouellic *et al.* (1990) *Proc. Natl. Acad. Sci. (USA)* **87**:4712; Shesely *et al.* (1991) *Proc. Natl. Acad. Sci. (USA)* **88**:4294). This approach is very effective when the number of cells receiving exogenous targeting transgene(s) is high (*i.e.*, with electroporation or with liposomes) and the treated cell populations are allowed to expand (Capecchi (1989)  
20 *Trends Genet.* **5**:70). The targeting construct may be introduced into the germline of an animal cell by methods including, *e.g.*, by pronuclear injection of recombinant genes into pronuclei of one-cell embryos, incorporating an artificial yeast chromosome into embryonic stem cells, gene targeting methods, or embryonic stem cell methodology. See, *e.g.*, U.S. Patent Nos. 4,736,866; 4,873,191; 4,873,316;  
25 5,082,779; 5,304,489; 5,174,986; 5,175,384; 5,175,385; 5,221,778; Gordon *et al.* (1980) *Proc. Natl. Acad. Sci. (USA)* **77**:7380-7384; Palmiter *et al.* (1985) *Cell* **41**:343-345; Palmiter *et al.* (1986) *Ann. Rev. Genet.* **20**:465-499; Askew *et al.* (1993) *Mol. Cell. Bio.* **13**:4115-4124; Games *et al.* (1995) *Nature* **373**:523-527; Valancius and Smithies (1991) *Mol. Cell. Bio.* **11**:1402-1408; Stacey *et al.* (1994) *Mol. Cell. Bio.*  
30 **14**:1009-1016; Hasty *et al.* (1995) *Nature* **350**:243-246; Rubinstein *et al.* (1993) *Nucl. Acid Res.* **21**:2613-2617.

Another method available for inserting a foreign nucleic acid sequence into the  $P_{2x}R$  gene locus to disrupt its expression is gene trapping. This method takes  
35 advantage of the cellular machinery present in all mammalian cells that splices exons into mRNA to insert a gene trap vector coding sequence into a gene in a random fashion. Once inserted, the gene trap vector creates a mutation in the trapped gene and disrupts its expression. By contrast to homologous recombination, this system of

5 mutagenesis is largely random. Thus, to obtain a genetically modified cell that contains the desired mutation of a P<sub>2x7</sub>R gene, this cell is typically selected from a pool of cells that contain mutations in a variety of genes.

Gene trapping systems and vectors have been described for use in genetically modified murine cells and other cell types (see, e.g., Allen *et al.* (1988) *Nature* 10 333:852-55; Bellen *et al.* (1989) *Genes Dev.* 3:1288-1300; Bier *et al.* (1989) *Genes Dev.* 3:1273-1287; Bonnerot *et al.* (1992) *J. Virol.* 66:4982-91; Brenner *et al.* (1989) *Proc. Nat. Acad. Sci. (USA)* 86:5517-21; Friedrich *et al.* (1993) *Meth. Enzymol.* 225:681-701; Friedrich *et al.* (1991) *Genes Dev.* 5:1513-23; Goff (1987) *Meth. Enzymol.* 152:469-81; Gossler *et al.*, (1989) *Science* 244:463-65; Hope (1991) 15 *Develop.* 113:399-408; Kerr *et al.* (1989) *Cold Spring Harb. Symp. Quant. Biol.* 2:767-776; Reddy *et al.* (1991) *J. Virol.* 65:1507-1515; Reddy *et al.* (1992) *Proc. Natl. Acad. Sci. (USA)* 89:6721-25; Skarnes *et al.* (1992) *Genes Dev.* 6:903-918; von Melchner *et al.* (1989) *J. Virol.* 63:3227-3233; and Yoshida *et al.* (1995) *Transgen. Res.* 4:277-87).

20 Promoter trap, or 5', vectors contain, in 5' to 3' order, a splice acceptor sequence followed by an exon typically characterized by a translation initiation codon and open reading frame and/or an internal ribosome entry site. In general, these promoter trap vectors do not contain promoters or operably linked splice donor sequence sequences. Consequently, after integration into the cellular genome of the 25 host cell, the promoter gene trap cassette intercepts the normal splicing of the upstream gene and acts as a terminal exon. Expression of the vector coding sequence is dependent upon the vector integrating into an intron of the disrupted gene in the proper reading frame. In such a case, the cellular splicing machinery splices exons from the trapped gene upstream of the vector coding sequence (Zambrowicz *et al.*, 30 WO 99/50426).

An alternative method for producing an effect similar to the above-described promoter trap vector is a vector that incorporates a nested set of stop codons present in, or otherwise engineered into, the region between the splice acceptor of the promoter trap vector and the translation initiation codon or polyadenylation sequence. 35 The coding sequence can also be engineered to contain an independent ribosome entry site (IRES) so that the coding sequence will be expressed in a manner largely

5 independent of the site of integration within the host cell genome. Typically, but not necessarily, an IRES is not used in conjunction with a nested set of stop codons.

Another type of gene trapping scheme uses a 3' gene trap vector. This type of vector contains, in operative combination, a promoter region that mediates expression of an adjoining coding sequence, the coding sequence, and a splice donor sequence  
10 that defines the 3' end of the coding sequence exon. After integration into a host cell genome, the transcript expressed by the vector promoter is spliced to a splice acceptor sequence from the trapped gene that is located downstream of the integrated gene trap cassette. Thus, the integration of the vector results in the expression of a fusion  
15 transcript comprising the coding sequence of the 3' gene trap cassette and any downstream cellular exons, including the terminal exon and its polyadenylation signal. When such vectors integrate into a gene, the cellular splicing machinery splices the vector coding sequence upstream of the 3' exons of the trapped gene. One advantage of such vectors is that the expression of the 3' gene trap vectors is driven by a promoter within the gene trap cassette and does not require integration into a  
20 gene that is normally expressed in the host cell (Zambrowicz *et al.*, WO 99/50426).

Examples of transcriptional promoters and enhancers that may be incorporated into the 3' gene trap cassette include, but are not limited to, cell or tissue specific promoters, inducible promoters, the herpes simplex thymidine kinase promoter, cytomegalovirus (CMV) promoter/enhancer, SV40 promoters, PGK promoter,  
25 regulatable promoters (*e.g.*, metallothioneine promoter), adenovirus late promoter, vaccinia virus 7.5K promoter, avian beta globin promoter, histone promoters (*e.g.*, mouse histone H3-614), beta actin promoter, and the cauliflower mosaic virus 35S promoter (see generally, Sambrook *et al.*, Molecular Cloning, Vols. I-III, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989), and Current  
30 Protocols in Molecular Biology, John Wiley & Sons (1989-2000 editions)).

The promoter gene trap or 3' gene trap cassette is introduced into target cells as a structural component of any of a wide range of vectors that can be specifically or nonspecifically inserted into the target cell genome. Suitable vectors that can be used in conjunction with the presently disclosed features include, but are not limited to,  
35 herpes simplex virus vectors, adenovirus vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, pseudorabies virus, alpha-herpes virus vectors, and the like. A thorough review of viral vectors, particularly viral vectors suitable for

5       modifying nonreplicating cells, and how to use such vectors in conjunction with the  
expression of polynucleotides of interest can be found in Viral Vectors: Gene  
Therapy and Neuroscience Applications, Editors Caplitt and Loewy, Academic Press,  
San Diego, 1995. Preferably, retroviral vectors are used for gene trapping. These  
vectors can be used in conjunction with retroviral packaging cell lines such as those  
10       described in U.S. Patent No. 5,449,614. Where non-murine mammalian cells are used  
as target cells for genetic modification, amphotropic or pantropic packaging cell lines  
can be used to package suitable vectors (Ory *et al.* (1996) *Proc. Natl. Acad. Sci. (USA)*  
**93**:11400-11406). Representative retroviral vectors that can be adapted to incorporate  
the presently described 3' gene trap cassette are described, for example, in U.S. Patent  
15       No. 5,521,076.

      The gene trapping cassettes can contain one or more of the heterologous  
marker genes discussed above with respect to targeting vectors used for homologous  
recombination. The marker gene may be engineered to contain an independent  
ribosome entry site (IRES) so that the marker is expressed in a manner largely  
20       independent of the location in which the vector has integrated into the target cell  
genome. Similar to their use in targeting vectors, markers are used in gene trapping  
vectors to select cells that have integrated the vector into the cell genome.

      Of course, gene trap vectors will integrate into the genome of infected host  
cells in a fairly random manner. Therefore, a genetically modified cell of the present  
25       invention which has a disrupted P<sub>2x7</sub>R gene must be identified from a population of  
cells that have undergone random vector integration. Preferably, the genetic  
modifications in the population of cells are of sufficient randomness and frequency  
such that the population represents mutations in essentially every gene found in the  
cell's genome, making it likely that a cell with a disrupted P<sub>2x7</sub>R gene will be  
30       identified from the population (see Zambrowicz *et al.*, WO 99/50426; Sands *et al.*,  
WO 98/14614).

      Individual mutant cells containing a disrupted P<sub>2x7</sub>R gene are identified in the  
population of mutated cells using, for example, reverse transcription and polymerase  
chain reaction (PCR) to identify a mutation in a P<sub>2x7</sub>R gene sequence. This process  
35       can be streamlined by pooling clones. For example, to find an individual clone  
containing a disrupted P<sub>2x7</sub>R gene, RT-PCR is performed using one primer anchored  
in the gene trap vector and the other primer located in the P<sub>2x7</sub>R gene sequence. A



5 positive RT-PCR result indicates that the vector sequence is encoded in the P<sub>2x7</sub>R gene transcript, indicating that P<sub>2x7</sub>R gene has been disrupted by a gene trap event (see, *e.g.*, Sands *et al.*, WO 98/14614).

For making non-human genetically modified animals containing one or more disrupted P<sub>2x7</sub>R genes, embryonic stem cells (ES cells) genetically modified by  
10 homologous recombination, gene trapping, or any other method known in the art, are preferred. Embryonic stem cells are manipulated according to published procedures (Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E. J. Robertson, ed., IRL Press, Washington, D.C. (1987); Zijlstra *et al.* (1989) *Nature* **342**:435-438; and Schwartzberg *et al.* (1989) *Science* **246**:799-803). Primary isolates of ES cells  
15 may be obtained directly from embryos, essentially as described for the EK.CCE cell line or for ES cells in general. The particular embryonic stem cell employed in the present invention is not critical. ES cells are preferably cultured on stromal cells, *e.g.*, STO cells and/or primary embryonic fibroblast cells as described by Robertson (1987) in Teratocarcinomas and Embryonic Stem Cells: a Practical Approach, E. J.  
20 Robertson, ed. (Oxford: IRL Press), pp. 71-112. The stromal (and/or fibroblast) cells serve to reduce the clonal outgrowth of abnormal ES cells.

The present invention encompasses ES cells transfected with a targeting construct and their progeny or potential progeny. While the ES cells of the invention are homozygous or heterozygous for the P<sub>2x7</sub>R disruption, they may not be identical to  
25 the parent ES cell transfected with the targeting construct due to mutations or environmental influences that may occur in other genes in succeeding generations.

Where the non-human genetically modified animal is a mouse, murine ES cells are used, such as AB-1 line grown on mitotically inactive SNL76/7 cell feeder layers (McMahon and Bradley (1990) *Cell* **62**:1073-1085). Other suitable ES lines  
30 include, but are not limited to, the E14 line (Hooper *et al.* (1987) *Nature* **326**: 292-295), the D3 line (Doetschman *et al.* (1985) *J. Embryol. Exp. Morph.* **87**:27-45), and the CCE line (Robertson *et al.* (1986) *Nature* **323**:445-448), the RW4 cell line (available from Genome Sciences), and the DBA cell line (Roach *et al.* (1995) *Exp. Cell Res.* **221**:520-525).

35 The P<sub>2x7</sub>R targeting transgene is transferred into mouse ES cells (*e.g.*, by electroporation) under conditions suitable for the continued viability of the electroporated ES cells. The electroporated ES cells are cultured under selective

5 conditions for positive selection (*e.g.*, a selective concentration of G418), and optionally are cultured under selective conditions for negative selection (*e.g.*, a selective concentration of gancyclovir or FIAU), either simultaneously or sequentially. Selected cells are then verified as having the correctly targeted transgene recombination by PCR analysis according to standard PCR or Southern blotting methods known in the art (see, *e.g.*, U.S. Patent No. 4,683,202; and Erlich *et al.* (1991) *Science* **252**:1643). Correctly targeted ES cells are then transferred into suitable blastocyst hosts for generation of chimeric genetically modified animals according to methods known in the art (Capecchi (1989) *Trends Genet.* **5**:70). The success of generating a mouse line from ES cells bearing a specific targeted mutation depends on the pluripotency of the ES cells (*i.e.*, their ability, once injected into a host blastocyst, to participate in embryogenesis and contribute to the germ cells of the resulting animal). The ES cells bearing a specific targeted mutation are injected into mouse blastocysts as described by Bradley, 1987, in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E. Robertson, ed. (Oxford: IRL Press), pp. 113-151. The particular mouse blastocysts employed in the present invention are not critical. Examples of such blastocysts include those derived from C57BL/6 mice, C57BL/6 Albino, Swiss outbred, CFLP, MFI, and the like. The blastocysts containing the injected ES cells are allowed to develop in the uteri of pseudopregnant non-human female mice and are born as chimeric mice. Chimeric targeted mice are derived according to Hogan *et al.*, Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; and Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E. J. Robertson, ed., IRL Press, Washington, D.C., 1987. The resultant genetically modified mice are chimeric for cells having an inactivated endogenous P<sub>2x7</sub>R loci and are backcrossed and screened for the presence of the correctly targeted transgene(s) by PCR or Southern blot analysis on tail biopsy DNA of offspring so as to identify genetically modified mice heterozygous for the inactivated P<sub>2x7</sub>R locus/loci. By performing the appropriate crosses, it is possible to produce a genetically modified non-mouse homozygous for a disrupted P<sub>2x7</sub>R locus (P<sub>2x7</sub>R<sup>-/-</sup>). P<sub>2x7</sub>R<sup>-/-</sup> genetically modified mice may also be crossed to mice carrying other mutations, such as P<sub>2x1</sub>R<sup>-/-</sup> mice.

In certain embodiments of the present invention, a disruption in the endogenous P<sub>2x7</sub>R gene locus in non-human genetically modified animals is limited to

5 specific developmental stages or to specific tissues. In other embodiments, a disruption in the endogenous  $P_{2x7}R$  gene locus in non-human genetically modified animals or cells derived from the non-human genetically modified animals is inducible.

10 Where the disruption in the  $P_{2x7}R$  gene locus is desired to be temporally or developmentally regulated, the Cre-Lox system may be employed. The Cre-Lox system may be used to activate or inactivate the  $P_{2x7}R$  gene at a specific developmental stage or in a particular tissue. Generally, methods utilizing Cre-Lox technology are carried out as described by Torres and Kuhn (1997) Laboratory  
15 Protocols for Conditional Gene Targeting, Oxford University Press. Methodology similar to that described for the Cre-Lox system can be employed utilizing the FLP-FRT system.

For inactivation of endogenous  $P_{2x7}R$  gene expression at a specific stage in development or a particular tissue, the  $P_{2x7}R$  coding region is replaced by a cassette comprising the coding region flanked by LoxP sites according to the methods  
20 described herein. The LoxP sites are targets for the Cre recombinase. The resulting genetically modified animal is crossed to another genetically modified animal in which the Cre recombinase is expressed under the control of a spatially and/or temporally regulated promoter. When Cre expression is activated, the LoxP sites undergo recombination to excise the endogenous  $P_{2x7}R$  coding region, resulting in  
25  $P_{2x7}R$ -deficient tissues.

For activation of endogenous  $P_{2x7}R$  gene expression in a selected tissue and or at a particular stage of development, the regions of homology in the targeting construct are promoter sequences, comprising an insertion fragment which contains multiple stop codons in all reading frames flanked by LoxP sites. Upon insertion of  
30 this targeting construct into the  $P_{2x7}R$  promoter, no  $P_{2x7}R$  protein is produced. The resulting genetically modified animal is crossed to another genetically modified animal in which the Cre recombinase is expressed under the control of a spatially and/or temporally regulated promoter. When Cre expression is activated, the LoxP sites undergo recombination to excise the stop codons and restore the  $P_{2x7}R$  gene to its  
35 undisrupted state.

For inducible  $P_{2x7}R$  activation or inactivation, the Tet operator is inserted into the endogenous  $P_{2x7}R$  regulatory elements, so that the  $P_{2x7}R$  gene falls under the

5 control of the tetracycline-controllable transactivator (tTA) and  
tetracycline-controllable repressor (TetR), which can only activate or repress  
transcription, respectively, in the presence of tetracycline. Genetically modified  
animals comprising the Tet promoter in the  $P_{2x7}R$  gene are then crossed to animals  
which express rTA or TetR, constitutively for example, and  $P_{2x7}R$  expression is  
10 induced or repressed by administering tetracycline to the animals. Alternatively,  
cultured cells from the genetically modified animals can be produced, the cells  
transfected with a rTA or TetR expression construct, and the culture contacted with  
tetracycline to induce or inhibit  $P_{2x7}R$  expression. For further details, see U.S. Patent  
No. 5,922,927.

15 The extent of  $P_{2x7}R$  deficiency resulting in a disruption in the endogenous  
 $P_{2x7}R$  gene loci or locus in non-human genetically modified animals and cells derived  
therefrom can easily be measured by using standard molecular biology methods. For  
instance, one can measure for a deficiency in  $P_{2x7}R$  messenger RNA levels by using  
reverse transcriptase mediated polymerase chain reaction (RT-PCR), Northern blot  
20 analysis, or *in situ* hybridization.

In other embodiments, the extent of  $P_{2x7}R$  deficiency resulting from a  
disruption in the endogenous  $P_{2x7}R$  gene loci or locus in a non-human genetically  
modified animal of the invention can be assayed by measuring protein levels or  
activity by various methods. For example, in one embodiment, protein extracts from  
25 cells having a disruption in the endogenous  $P_{2x7}R$  gene loci or locus-deficient cells  
and tissues are assayed for their levels of  $P_{2x7}R$  protein by affinity to various  
immunoassays known in the art. Such immunoassays include but are not limited to  
competitive and non-competitive assay systems using techniques such as  
radioimmunoassays (RIA), enzyme linked immunosorbent assay (ELISA), "sandwich"  
30 immunoassays, immunoradiometric assays, gel diffusion precipitin reactions,  
immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or  
radioisotope labels, for example), Western blots, precipitation reactions,  
immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*  
In one mode of the embodiment, antibody binding is detected by detecting a label on  
35 the primary antibody. In another mode of embodiment, the primary antibody is  
detected by detecting binding of a secondary antibody or reagent to the primary  
antibody. Optionally, the secondary antibody can be labeled. Many means are known

5 in the art for detecting binding in an immunoassay and are within the scope of the present invention.

Methods of measuring the P<sub>2x7</sub>R-like activities are described herein in the Example below and in the literature. Examples of such methods are as follows: ATP-induced changes in cell permeability can be measured by cellular electrophysiological  
10 changes and/or the cellular accumulation of macromolecules such as dyes (see, *e.g.*, Michel *et al.* (1998) *Br. J. Pharmacol.* **124**:1194-1201; Virginio *et al.* (1999) *J. Physiol.* **519**:335-346; and the Examples disclosed herein); changes in ATP-induced posttranslational processing and production of interleukins can be measured using immunoprecipitation and/or ELISA assays (*e.g.*, as further described in the  
15 Example below); and changes in lymphoproliferation can be measured as reported in Baricordi *et al.* (*J. Biol. Chem.* (1999) **274**:33206-33208).

Techniques known to those of skill in the art can be used to measure cell proliferation. For example, cell proliferation can be measured by counting samples of a cell population over time (*e.g.* daily cell counts). Cells can be counted using a  
20 hemacytometer and light microscopy (*e.g.*, HyLite hemacytometer, Hausser Scientific). Cell number can be plotted against time in order to obtain a growth curve for the population of interest. In a preferred embodiment, cells counted by this method are first mixed with the dye Trypan-blue (Sigma, St. Louis, MO), such that living cells exclude the dye, and are counted as viable members of the population.  
25 Cell proliferation can also be measured using (<sup>3</sup>H)-thymidine incorporation (see, *e.g.*, Chen (1996) *Oncogene* **13**:1395-403; Jeoung (1995) *J. Biol. Chem.* **270**:18367-73). This assay allows for quantitative characterization of S-phase DNA synthesis. In this assay, cells synthesizing DNA incorporate (<sup>3</sup>H)-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques in the art such as  
30 by counting of radioisotope in a Scintillation counter (*e.g.*, Beckman LS 3800 Liquid Scintillation Counter).

The cells from a non-human genetically modified animal can be isolated from tissue or organs using techniques known to those of skill in the art. For example, immune cells can be collected or isolated from blood, or secondary lymphoid organs  
35 of the subject such as, but not limited to, lymph nodes, tonsils, the spleen, Peyer's patch of the intestine, and bone marrow, by any of the methods known in the art (see,

5     *e.g.*, Current Protocols in Immunology (1991) Green Publishing Associates, New  
York, NY, p. 21). Immune cells obtained from such sources typically comprise  
predominantly recirculating lymphocytes and macrophages at various stages of  
differentiation and maturation. Optionally, standard techniques, such as  
morphological observation and immunochemical staining, can be used, if desired, to  
10    verify the presence of the desired cells, *e.g.*, dendritic, T cells, and macrophages. The  
immune cells used in the *in vitro* methods of the invention can be collected by  
standard techniques, such as by use of a syringe to withdraw the blood, followed by  
subjecting the blood to Ficoll-Hypaque (Pharmacia) gradient centrifugation. Blood,  
anticoagulated with preservative-free heparin, usually yields  $0.5-1.0 \times 10^6$   
15    lymphocytes/ml. Separated blood cells (*e.g.*, macrophages) may be frozen by standard  
techniques prior to use in the present methods. In a specific embodiment, the immune  
cells used are purified white blood cells comprising lymphocytes and macrophages.

Monocyte-derived macrophages can be isolated from immune cells by  
allowing the cells to adhere to culture flasks for 1-2 hours, washing away nonadherent  
20    cell and culturing adherent cells for 7-12 days in medium (*i.e.*, RPMI 1640  
supplemented with 20% human serum, 2 mM glutamine, 5 mM HEPES, and 100  
 $\mu\text{g/ml}$  streptomycin) plus interferon- $\gamma$  (IFN- $\gamma$ ). See, *e.g.*, Blanchard *et al.* (1995)  
*J. Cell. Biochem.* 57:452-464; and Blanchard *et al.* (1991) *J. Immunol.* 147:2579-  
2585.

25         In one embodiment, antibodies against specific surface markers can be directly  
labeled by conjugation of an affinity compound to such antibodies to facilitate  
detection and separation of macrophages or lymphocytes. Alternatively, in another  
embodiment, the primary antibody is detected by detecting binding of a secondary  
antibody or reagent to the primary antibody. In a further embodiment, the secondary  
30    antibody is labeled.

Affinity compounds that can be used include, but are not limited to, biotin,  
photobiotin, fluorescein isothiocyanate (FITC), or phycoerythrin (PE), or other  
compounds known in the art. Cells retaining labeled antibodies are then separated  
from cells that do not bind such antibodies by techniques known in the art such as, but  
35    not limited to, various cell sorting methods (*e.g.*, fluorescence activated cell sorter

5 (FACS) or magnetic activated cell sorting (MACS), affinity chromatography, and panning).

In one embodiment, cells isolated from a non-human genetically modified animal of the invention are immortalized. In accordance with this embodiment, cells derived from the non-human genetically modified animal can be immortalized by  
10 genetically engineering the telomerase gene, an oncogene, *e.g.*, *mos* or *v-src*, or an apoptosis-inhibiting gene, *e.g.*, *bcl-2*, into the cells. Alternatively, cells derived from the non-human genetically modified animal can be immortalized by fusion with a hybridization partner utilizing techniques known to one of skill in the art.

The present invention encompasses cells isolated from a non-human,  
15 genetically modified animal and their progeny or potential progeny. Progeny may not be identical to the parent cells isolated from the non-human, genetically modified animal due to mutations or environmental influences that may occur in succeeding generations.

The present invention encompasses the identification of genes whose  
20 expression is upregulated in  $P_{2x7}R^{+/+}$  or  $P_{2x7}R^{-/-}$  non-human mammals or animal cells relative to their respective control. Techniques known to those of skill in the art can be used to identify such genes. For example, DNA assays can be used to identify genes whose expression is upregulated in  $P_{2x7}R^{+/+}$  or  $P_{2x7}R^{-/-}$  mice to compensate for a deficiency in  $P_{2x7}R$  expression. DNA arrays are known to those of skill in the art.  
25 See, *e.g.*, U.S. Patent No. 5,965,352; Schena *et al.* (1995) *Science* **270**:467-470; DeRisi *et al.* (1996) *Nature Genetics* **14**:457-460; Shalon *et al.* (1996) *Genome Res.* **6**:639-645; and Schena *et al.* (1995) *Proc. Natl. Acad. Sci. (USA)* **93**:10539-11286.

In another application, the genetically-modified  $P_{2x7}R^{-/-}$  non-human mammals and animal cells of the invention can be used to determine whether the  $P_{2x7}R$  plays a  
30 role in causing symptoms to develop in certain models of disease, such as inflammatory diseases and neurodegenerative diseases. For example, if a symptom of inflammation or neurodegeneration is reduced or absent in a  $P_{2x7}R^{-/-}$  non-human mammal or animal cell under conditions that induce such a symptom in a corresponding  $P_{2x7}R^{+/+}$  or  $P_{2x7}R^{+/-}$  non-human mammal or animal cell, then the  $P_{2x7}R$   
35 plays a role in curbing the symptoms. Inflammation can be assessed in wild type mice,  $P_{2x7}R^{+/-}$  mice, and  $P_{2x7}R^{-/-}$  mice following the onset of arthritis induced, *e.g.*, by administering collagen or collagen fragments (Joosten *et al.* (1999) *J. Immunol.*

5      163:5049-55), by administering collagen monoclonal antibodies (Terato *et al.* (1992) *J. Immunol.* **148**:2103-08), or by administering zymosan (Van de Loo *et al.* (1995) *Arthritis Rheuma.* **38**:164-72). The inflammation can be detected using any method known to those of skill in the art. For example, inflammation can be detected histologically by computed tomography (CT), magnetic resonance imaging (MRI),  
10      ultrasonography, scintigraphic imaging, or by visual observation of symptoms such as swelling or redness.

Such comparison can also be made in models of neurodegeneration. Alzheimer's disease can be induced in a mouse, for example, by expressing human familial Alzheimer's disease (FAD)  $\beta$ -amyloid precursor (APP) in the mouse, overexpressing human wild-type APP in the mouse, overexpressing  $\beta$ -amyloid 1-42  
15      ( $\beta$ A) in the mouse, or expressing FAD presenillin-1 (PS-1) in the mouse. (*See, e.g., Higgins (1999) Mol. Med. Today* **5**:274-276.) Gene products with altered concentrations in genetically modified mice with Alzheimer's disease which can be measured include, but are not limited to, amyloid  $\beta$ -peptides ( $A\beta$ ), tau protein, and neuronal thread protein (NTP) in the CSF of the mouse. Stroke can be induced in  
20      mouse, for example, by middle cartoid artery occlusion (*see, e.g., Garcia et al. (1995) Am. J. Pathol.* **147**:1477-86; Hara *et al. (1997) Proc. Natl. Acad. Sci. (USA)* **94**:2007-12).

Under circumstances in which an agent has been identified as a  $P_{2x7}R$  agonist or antagonist (*e.g., the agent significantly modifies one or more of the  $P_{2x7}R$  polypeptide activities described herein when the agent is administered to a  $P_{2x7}R^{+/+}$  or  $P_{2x7}R^{-/-}$  non-human mammal or animal cell*), the genetically-modified,  $P_{2x7}R^{-/-}$  animal cells of the invention are useful to characterize any other effects caused by the agent besides those known to result from the (ant)agonism of a  $P_{2x7}R$ . For example, if  
25      the administration of the agent causes an effect in a  $P_{2x7}R^{+/+}$  non-human mammal or animal cell that is not known to be associated with  $P_{2x7}R$  polypeptide activity, then one can determine whether the agent exerts this other effect solely or primarily through modulation of the  $P_{2x7}R$  by administering the agent to a corresponding  $P_{2x7}R^{-/-}$  non-human mammal or animal cell. If this other effect is absent, or is significantly  
30      reduced, in the  $P_{2x7}R^{-/-}$  non-human mammal or animal cell, then the other effect is mediated, or at least in part, by the  $P_{2x7}R$ . However, if the  $P_{2x7}R^{-/-}$  non-human mammal or animal cell exhibits the other effect to a degree comparable to the  $P_{2x7}R^{+/+}$   
35



5 or P<sub>2x7</sub>R<sup>+/−</sup> non-human mammal or animal cell, then the other effect is mediated by a pathway that does not involve P<sub>2x7</sub>R signaling.

Examples of agents that may be identified as P<sub>2x7</sub>R antagonists or agonists include, but are not limited to, nucleic acids (*e.g.*, DNA and RNA), carbohydrates, lipids, proteins, peptides, peptidomimetics, small molecules, and other drugs that  
10 modulate a P<sub>2x7</sub>R-like activity in an animal cell. Such agents may be identified from libraries of agents that are obtained using any methods such as the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound"  
15 library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (*e.g.*, Lam (1997) *Anticancer Drug Des.* **12**:145; U.S. Patent No. 5,738,996; and U.S. Patent No. 5,807,683).

20 Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. (USA)* **90**:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. (USA)* **91**:11422; Zuckermann *et al.* (1994) *J. Med. Chem.* **37**:2678; Cho *et al.* (1993) *Science* **261**:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* **33**:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* **33**:2061; and Gallop *et al.* (1994) *J. Med. Chem.* **37**:1233.

25 Libraries of agents may be presented in solution (*e.g.*, Houghten (1992) *Bio/Techniques* **13**:412-421), or on beads (Lam (1991) *Nature* **354**:82-84), chips (Fodor (1993) *Nature* **364**:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. (USA)* **89**:1865-1869) or phage (Scott and Smith (1990) *Science* **249**:386-390; Devlin (1990) *Science* **249**:404-406; Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. (USA)* **87**:6378-6382; and Felici (1991) *J. Mol. Biol.* **222**:301-310).

30 Agents that modulate P<sub>2x7</sub>R activity can be used to treat, inhibit, or prevent certain diseases and disorders associated with aberrant P<sub>2x7</sub>R expression or activity. Such diseases and disorders include, but are not limited to, glomerular diseases, chronic inflammatory diseases (*e.g.*, arthritis, including rheumatoid arthritis and osteoarthritis, bacterial infection, psoriasis, septicemia, cerebral malaria, inflammatory

5 bowel disease, gastroenteritis, glomerular nephritis, asthma, allergic rhinitis, contact dermatitis, and toxic shock), and neurodegenerative disorders (*e.g.*, Alzheimer's disease, dementia, Parkinson's disease, stroke, Huntington's disease, multiple sclerosis, multiple pontine myelinolysis, human immunodeficiency associated myelopathy, and transverse myelopathy).

## 10 EXAMPLES

The following examples are provided for illustrative purposes only and are not to be construed as limiting the present invention in any manner.

### 15 I. MATERIALS AND METHODS

#### Construction of the P<sub>2x7</sub>R Targeting Vector and Generation of the Knockout Mice

20 A cDNA probe specific for the mouse P<sub>2x7</sub>R gene was prepared by reverse transcription and PCR amplification using oligonucleotide primers P<sub>2x7</sub>R-F1 (5'CGG CGT GCG TTT TGA CAT CCT 3'; SEQ ID NO:1) and P<sub>2x7</sub>R-R2 (5' AGG GCC CTG CGG TTC TC 3'; SEQ ID NO:2) which were designed based on the published  
25 sequence of the rat sequence of the P<sub>2x7</sub>R gene (Surprenant *et al.* (1996) *Science* **272**:735-738). Total RNA isolated from the J774 A 1 mouse monocyte/macrophage cell line (American Type Culture Collection, Manassas, VA) was used as the template RNA. This PCR product was 401 bp long and was cloned and sequenced to verify that  
30 it corresponded to the mouse P<sub>2x7</sub>R gene. The probe was used to screen a 129/Sv mouse genomic library (Stratagene, La Jolla, CA), and to isolate a single positive genomic clone. Sequence analysis of *Bam*HI sub-cloned fragment confirmed that this clone corresponded to the mouse P<sub>2x7</sub>R gene. A targeting vector was constructed which inserted the neomycin resistance gene from the pJNS2 plasmid (Dombrowicz *et al.* (1993) *Cell* **75**:969-76) directly after the Arg<sup>505</sup> codon deleting Cys<sup>506</sup> - Pro<sup>532</sup> in  
35 the carboxy region of the P<sub>2x7</sub>R gene, based on the published sequence (Chessell *et al.* (1998) *FEBS Lett.* **4390**:260-300). The upstream and downstream regions of homology were each approximately 5-8 Kb in length (Figure 1). One of these two

5 fragments corresponding to the region of the P<sub>2x7</sub>R gene immediately 5' of the Arg<sup>505</sup>  
and the second DNA fragment corresponding to the DNA located 3' of codon Pro<sup>532</sup>  
were cloned on either side of the *neo* gene present in the JNS2 plasmid. These  
fragments direct the homologous integration of the targeting vector with the  
endogenous P<sub>2x7</sub>R gene in the embryonic stem cell line E14TG2a (Hooper et al.  
10 (1987) *Nature* 326:292-5). 129/Ola derived E14Tg2a embryonic stem (ES) cells  
(American Type Culture Collection, Manassas, VA) (Hooper et al. (1987) *Nature*  
326:292-5) were grown, transformed and screened using standard methods (Mohn *et*  
*al.* In: *DNA Cloning* 4, pp. 143-184, Oxford Press, New York, (1995)). Homologous  
recombination events between the vector and ES cells and genotyping of mice was  
15 detected using a probe which hybridizes to a 16 Kb band of the wild type P<sub>2x7</sub>R locus  
and a 10 Kb band in the targeted locus (Figure 2A). This probe was derived from the  
genomic DNA fragment isolated from the 129/SV library using the P<sub>2x7</sub>R-specific  
probe. This fragment of DNA corresponds to the region of the P<sub>2x7</sub>R gene just 5' of  
the region of the gene used in the construction of the targeting vector. This probe  
20 detects a 16 Kb fragment in the wild type and 10 Kb DNA fragment from the targeted  
allele. Chimeric mice derived from targeted ES cells were mated with B6D2  
(C57BL/6 x DBA/2F1) or C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) to  
produce heterozygous animals which were then bred to produce homozygous mutant  
animals. Analysis of RNA prepared from cultured bone marrow derived mast cells  
25 from P<sub>2x7</sub>R<sup>+/+</sup> and P<sub>2x7</sub>R<sup>-/-</sup> mice with P<sub>2x7</sub>R specific probes verified the loss of  
expression in P<sub>2x7</sub>R<sup>-/-</sup> mice (Figure 2B).

#### Peritoneal Macrophage Isolation

30 Peritoneal macrophages were harvested by injecting 5 ml of Roswell Park  
Memorial Institute (RPMI) medium containing 5% FBS into each peritoneal cavity,  
immediately prior to injection, the animals were sacrificed by cervical dislocation.  
The injected medium was dispersed throughout the peritoneal cavity by rubbing the  
external surface, after which a hole in the skin covering the peritoneum was made to  
35 gain access to the cavity and the injected fluid was recovered with the aid of a transfer  
pipet. Lavage fluids from multiple animals were pooled and the cells were collected  
by centrifugation (300 x g). These cell pellets were washed twice by centrifugation in

5        RPMI containing 5% fetal calf serum (FCS). A cell count was performed before the final wash.

#### Western Analysis

10            Peritoneal macrophage cell pellets were washed once in Cavitation Buffer (25 mM Hepes, pH 7, 30 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 ug/ml leupeptin, and 1 µg/ml pepstatin) by centrifugation. Cell pellets then were suspended in 2.5 ml of Cavitation Buffer and the cells were disrupted by nitrogen cavitation (15 minutes on ice at 750 psi). The resulting cell lysates were adjusted to 0.1%  
15        saponin, incubated on ice for 30 minutes, and cell membranes subsequently were recovered by centrifugation (50,000 rpm for 30 minutes at 4°C in a Beckman Ti70 rotor). The membrane pellet was suspended in 2 ml Cavitation Buffer with the aid of a glass tube-teflon pestle homogenizer, and an aliquot of the suspension was set aside for analysis of total protein (Pierce Assay; Pierce, Rockford, IL). The membranes  
20        again were collected by centrifugation after which the pellets were suspended in 100 µl of 2x Laemmli sample buffer.

             Forty mg of protein was loaded into wells of a 4-20% Tris-Glycine gel (Novex, San Diego, CA), and, after separation of the proteins, they were transferred to nitrocellulose. These blots were blocked overnight at 4°C in 1X Western Blocking  
25        Reagent (Boehringer Mannheim, Indianapolis, IN) in TBS-T (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween-20). Blots then were incubated for 2 hours at room temperature in a TBS-T solution containing a 1 to 200 dilution of anti-P<sub>2x7</sub>R serum (Alomone, Jerusalem, Israel) and 1x Western Blocking Reagent. Blots were washed in TBS-T (two rinses, 15 minutes each), and then incubated for 1 hour with TBS-T  
30        containing a 1 to 2000 dilution of horseradish peroxidase- (HRP-)conjugated anti-rabbit IgG-(NE BioLabs; Beverly, MA) and 1x Western Blocking Reagent. Blots were washed in TBS-T (two rinses, 15 min each), and then developed with Super Signal (Pierce, Rockford, IL) and imaged with a Lumi-Imager (Boehringer Mannheim).

35

5                    YoPro Yellow Uptake

Peritoneal macrophages were washed with Isotonic Medium (15 mM Hepes, pH 7.2, 135 mM NaCl, 5 mM KCl, 18 mM CaCl<sub>2</sub>, 08 mM MgCl<sub>2</sub>) by centrifugation and the resulting cell pellet was suspended in Isotonic Medium to achieve a final cell  
10 concentration of 1x10<sup>6</sup> cells/ml. Fifty µl of this cell suspension then was placed into wells of a Microfluor "B" U-bottom plate (Dynatech, Chantilly, VA), and 50 µl of 2 µM YoPro Yellow (Molecular Probes, Eugene, OR); the fluorescent dye was dissolved in Isotonic Medium. Wells were then adjusted to 5 mM ATP or 0.0075% saponin by addition of concentrated stock solutions of these agents. Fluorescence was  
15 monitored as a function of time at 37°C, excitation, 450 nm/emission, 530nm.

Stimulus-Induced IL-1β Posttranslational Processing *In Vitro*

Macrophages from wild type and P<sub>2x7</sub><sup>-/-</sup> animals (1 x 10<sup>6</sup> cells seeded per well  
20 of 6-well cluster plates) were stimulated with 1 µg/ml *E. coli* LPS (Serotype 055 B5 obtained from Sigma; St. Louis, MO) for 75-90 minutes and then rinsed with 2 ml of methionine-free RPMI medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 1% dialyzed FBS, 1 µg/ml LPS, and 25 mM Hepes, pH 7.3 (Pulse Medium). One ml of Pulse Medium containing 83 µCi/ml of <sup>35</sup>S-methionine  
25 (Amersham Corp, Chicago, IL) then was added to each well, and the cells were labeled at 37° C for 1 hour. These labeled cells subsequently were rinsed twice with RPMI 1640 medium containing 100 units/ml penicillin, 100 µg/ml streptomycin, 1% FBS, 2 mM glutamine, 1 µg/ml LPS and 25 mM Hepes, pH 7.3 (Chase Medium). One ml of Chase Medium (containing no effector (control) or containing 05 mM ATP,  
30 or 20 mM nigericin) was then added to each well, and the cells were chased at 37°C for 30 minutes. Media were harvested and clarified by centrifugation (6000 x g for 5 minutes) to remove cells and/or cell debris. Cell monolayers were suspended in 1 ml of a lysis buffer composed of 1% Triton X-100, 150 mM NaCl, 25 mM Hepes, pH 7, 0.1 mM PMSF, 1 mg/ml ovalbumin, 1 mM iodoacetic acid, 1 µg/ml pepstatin, and 1  
35 µg/ml leupeptin. Clarified media samples were adjusted to the same final Triton X-100 and protease inhibitor concentrations by addition of aliquots from concentrated stocks of these reagents. After a 30 minute incubation on ice, all samples were

5 clarified by centrifugation at 45,000 rpm for 30 minutes in a TLA-45 rotor (Beckman,  
Palo Alto, CA), the resulting supernatants were recovered, and IL-1 $\beta$  was  
immunoprecipitated from these samples using a goat anti-murine IL-1 $\beta$  serum  
obtained from Dr. Ivan Otterness (Pfizer Central Research, Groton, CT).  
Immunoprecipitates were fractionated by SDS gel electrophoresis, the quantity of  
10 radioactivity associated with individual IL-1 $\beta$  polypeptide species was determined by  
scanning dried gels with a Phosphoimager.

#### ATP-Induced IL-1 $\beta$ Posttranslational Processing *In Vivo*

15 Groups of mice were injected intraperitoneally (ip) with 1  $\mu$ g of LPS. Two  
hours after this LPS injection, mice were injected ip with either 30 mM ATP (adjusted  
to pH 7) or PBS. Mice were sacrificed 30 minutes or 120 minutes after the ATP or  
PBS injection and peritoneal cavities were lavaged, each with 3 ml of RPMI (5%  
FCS). Samples of peritoneal lavages were spun down, supernatants were collected  
20 and tested for the presence of IL-1 $\beta$  and IL-6 using ELISAs (Amersham Life Sciences,  
Arlington Heights, VA and Endogen, Woburn, MA, respectively).

## II. RESULTS

### 25 Generation and Characterization of P<sub>2x7</sub>R<sup>-/-</sup> Mice

Mouse ES cells in which the P<sub>2x7</sub>R gene was disrupted by homologous  
recombination were generated using the scheme shown in Figure 1. The targeting  
vector was constructed such that the neomycin-resistance gene was inserted into an  
exon encoding the carboxy terminus of the wild-type polypeptide. Integration of the  
30 targeting vector into the mouse genome by homologous recombination results in  
replacement of the region of the gene encoding Cys<sup>506</sup> to Pro<sup>532</sup> with the neomycin  
resistance gene. ES cells containing the mutant P<sub>2x7</sub>R allele were identified by  
Southern blot analysis and used to generate the P<sub>2x7</sub>R <sup>$\Delta$ 506-532</sup> mouse line. Targeted ES  
cells contributed to the germline of chimeric mice, and F1 generation <sup>+/+</sup> animals  
35 appeared normal. Homozygous null animals were recovered in the F2 generation with  
the expected Mendelian frequencies. Southern blot analysis of <sup>+/+</sup> and <sup>+/+</sup> animals

5 confirmed the success of the targeting strategy. The wild-type and knockout alleles appeared as a 16 kbp and 10 kbp restriction fragments, respectively, on Southern blots (Figure 2A). Heterozygous ( $^{+/-}$ ) animals demonstrated the presence of both alleles, but the knockouts contained only the targeted allele (Figure 2A). Northern analysis of RNA isolated from cultured bone marrow mast cells signified that the transcript for the  $P_{2x7}R$  was present in the wild-type ( $^{+/+}$ ) but not knockout ( $^{-/-}$ ) animals (Figure 2B).  
10  $P_{2x7}R$  knockout animals were viable and fertile, and demonstrated no gross abnormalities.

To demonstrate that the knockout animals lacked the  $P_{2x7}$  receptor ( $P_{2x7}R$ ), membranes from peritoneal macrophages were compared by Western analysis for the presence of the receptor polypeptide. Membranes from wild-type peritoneal  
15 macrophages contained a 76 kDa polypeptide that cross reacted with the  $P_{2x7}R$  antiserum. The size of the murine polypeptide is comparable to that displayed by the human  $P_{2x7}R$  when over expressed in HEK293 cells (Figure 3). In contrast, macrophage membranes prepared from cells isolated from the knockout animals did not contain a similarly sized polypeptide (Figure 3).  
20

A hallmark of the  $P_{2x7}R$  is its ability to facilitate translocation of large organic molecules such as the fluorescent dye YoPro Yellow in response to ATP activation (Virginio et al. (1999) *J. Physiol.* **519**:335-346). When mouse peritoneal macrophages isolated from wild-type animals were activated with 5 mM ATP in the presence of extracellular YoPro Yellow, a time dependent increase in fluorescence  
25 intensity was observed (Figure 4). This increase in fluorescence results from internalization of the dye molecules followed by their binding to DNA; when bound to DNA, the fluorescence intensity increases. In the absence of ATP, no significant increase in fluorescence intensity was observed, indicating that YoPro Yellow is impermeant to the plasma membrane in the absence of the nucleotide triphosphate. In contrast, addition of ATP to macrophages isolated from  $P_{2x7}R^{-/-}$  mice did not result in time-dependent increase in fluorescence intensity (Figure 4A). Macrophages isolated from the  $P_{2x7}R^{-/-}$  mice demonstrated the same low fluorescence in the absence and  
30 presence of extracellular ATP. In the presence of saponin, a detergent that

5 permeabilizes the plasma membrane, YoPro Yellow accumulates to the same extent in the both wild-type and  $P_{2x7}R^{-/-}$  macrophages (Figure 4B). Therefore, the knockout of the  $P_{2x7}R$  is associated with loss of ATP-dependent YoPro Yellow accumulation.

#### Stimulus-Coupled IL-1 $\beta$ Posttranslational Processing

10 Peritoneal macrophages isolated from wild-type and  $P_{2x7}R^{-/-}$  mice were stimulated with LPS and labeled with [ $^{35}S$ ]methionine. These radiolabeled cells then were cultured in the absence or presence of a secretory stimulus, after which cells and media were harvested separately, cells were solubilized by detergent extraction, and  
15 IL-1 $\beta$  was recovered from the media and cell extracts by immunoprecipitation. Radiolabeled 35 kDa proIL-1 $\beta$  was recovered from cell extracts derived from both wild-type and  $P_{2x7}R^{-/-}$  macrophages (Figures 5A and 5B). The amount of [ $^{35}S$ ]methionine recovered as the cell-associated 35 kDa polypeptide after the chase in the absence of a secretory stimulus was 43,900 PSL/LDH eq and 44,100 PSL/LDH eq,  
20 respectively, from the wild-type and knockout macrophages. This similarity suggests that the two cell-types generated comparable amounts of proIL-1 $\beta$  in response to LPS activation. Neither the wild-type nor the  $P_{2x7}R^{-/-}$  macrophages released radiolabeled IL-1 $\beta$  to the medium in the absence of a secretory stimulus (Figure 5B).

Treatment of LPS-activated/[ $^{35}S$ ]methionine-labeled wild-type macrophages  
25 with extracellular ATP promoted formation and release of 17 kDa IL-1 $\beta$  (Figure 5B). Cytokine that remained cell-associated was, for the most part, recovered as the 35 kDa procytokine (Figure 5A). After correcting for the 2-fold loss of radioactivity that occurs when the 35 kDa procytokine is proteolytically processed by caspase-1, the extracellular 17 kDa polypeptide represents 64% of the total (sum of cell-associated and media species) radiolabeled IL-1 $\beta$  recovered from these cultures (Figure 6A). In  
30 sharp contrast, LPS-activated/[ $^{35}S$ ]methionine-labeled  $P_{2x7}R^{-/-}$  macrophages did not release any radiolabeled IL-1 $\beta$  to the medium in response to ATP (Figure 5B). Moreover, cytokine recovered from extracts of the ATP-treated  $P_{2x7}R^{-/-}$  macrophages persisted as the 35kDa procytokine species (Figure 5A). ATP-treated wild-type, but  
35 not knockout, macrophages demonstrated enhanced release of cytoplasmic enzyme lactate dehydrogenase (LDH) relative to non-ATP treated cultures (Figure 6B).



5 In response to the potassium ionophore nigericin, on the other hand, both wild-type and  $P_{2x7}R^{-/-}$  macrophages produced and released 17 kDa IL-1 $\beta$  (Figure 5B). The extracellular mature cytokine represented 66% and 74%, respectively, of the total [ $^{35}S$ ]methionine-labeled IL-1 $\beta$  recovered from the wild-type and  $P_{2x7}R^{-/-}$  cultures (Figure 6A). Moreover, both macrophage populations released comparable quantities of LDH in response to nigericin (Figure 6B). Thus,  $P_{2x7}R^{-/-}$  macrophages produce proIL-1 $\beta$  in response to LPS challenge, and they posttranslationally process this procytokine in response to nigericin stimulation. However, as a result of the absence of the  $P_{2x7}R$ , these cells do not produce or release mature IL-1 $\beta$  in response to ATP challenge.

#### 15 Characterization of *In Vivo* Cytokine Production Capabilities

Peritoneal macrophages exposed to LPS *in vivo* also require a secretion stimulus to elicit efficient externalization of mature IL-1 $\beta$  (Griffiths *et al.* (1995) *J. Immunol.* **154**:2821-2828). To determine whether absence of the  $P_{2x7}R$  affects this behavior, mice were primed with LPS, and 2 hours later they received an ip injection of PBS with or without ATP. Peritoneal lavage fluids from these mice then were assessed for IL-1 $\beta$  content by ELISA. Wild-type LPS-primed animals yielded no significant IL-1 $\beta$  in response to PBS (Figure 7), but abundant quantities of IL-1 $\beta$  were detected following ATP challenge (Figure 7). A similar pattern was observed in mice heterozygous for the  $P_{2x7}R$  disruption (+/-) (Figure 7). In contrast, LPS-primed  $P_{2x7}R^{-/-}$  mice failed to generate significant levels of IL-1 $\beta$  in response to ATP challenge (Figure 7).

As noted earlier, IL-1 signaling often leads to the production of other cytokines such as IL-6 (Allen *et al.* (2000) *J. Exp. Med.* **191**:859-869). To demonstrate this cascade effect, mice were primed by an injection of LPS (or phosphate buffered saline) at time 0. Two hours later an injection of ATP (or PBS) was administered to promote IL-1 posttranslational processing and the mice were incubated for an additional 4 hours. Peritoneal lavages were collected at several times during the course of the experiment and analyzed for IL-6 by ELISA. Samples recovered from wild-type mice subjected to an initial priming injection of PBS followed 2 hours later by ATP did not contain significant levels of IL-6 at any time (Figure 8). Mice that

5 received an initial priming injection of LPS followed by PBS yielded levels of IL-6 at  
1 and 2 hours (4 to 5 ng/ml) that were elevated above those recovered from mice  
injected with the combination of phosphate buffered saline and ATP (Figure 8). By  
4 hours, however, IL-6 levels recovered from the LPS-PBS treated animals returned to  
baseline (Figure 9). On the other hand, LPS-primed mice that subsequently were  
10 challenged with ATP demonstrated a dramatic increase in lavage fluid IL-6, reaching  
a peak value >25 ng/ml at the 4 hour time point (Figure 8).

Wild-type and  $P_{2x7R}^{-/-}$  mice were compared in this two stage production assay  
format to determine whether absence of the receptor altered IL-6 production. Mice  
were administered a priming ip injection of LPS followed 2 hours later by a PBS or  
15 ATP challenge. Peritoneal lavage fluids subsequently were collected at 30 and 120  
minutes and these were assessed for cytokine content by ELISA. Wild-type mice  
primed with an intraperitoneal injection of LPS and challenged with an intraperitoneal  
injection of PBS or ATP for 30 minutes yielded 0.02 ng/ml and 0.4 ng/ml of IL-1 $\beta$ ,  
respectively (Table 1). When the time of the challenge reaction was extended to  
20 2 hours, IL-1 $\beta$  levels declined slightly to 0.34 ng/ml in the ATP-treated mice, but  
levels of IL-1 $\beta$  within lavage fluids recovered from PBS challenged mice remained at  
the lower limit of detection (Table 1). Minimal levels of IL-1 $\beta$  also were recovered  
from  $P_{2x7R}^{-/-}$  mice challenged with PBS, and ATP challenge did not augment  
production of IL-1 $\beta$  by these mice at either the 30 minute or 120 minute time point  
25 (Table 1). Quantities of IL-6 generated by wild-type and knockout animals in  
response to PBS challenge were comparable at the 30 minute harvest, representing  
6 ng/ml and 5 ng/ml, respectively (Table 1). After 120 minutes of PBS challenge, IL-  
6 levels declined in both sets of animals to baseline values (Table 1). After 30  
minutes of ATP challenge, both wild-type and  $P_{2x7R}^{-/-}$  mice yielded 8 to 9 ng/ml of  
30 IL-6; these values are slightly elevated over the quantities of IL-6 recovered from the  
PBS challenged animals at 30 minutes (Table 1). After 120 minutes of ATP  
challenge, on the other hand, wild-type animals yielded greater levels of IL-6  
(17 ng/ml) than did the  $P_{2x7R}^{-/-}$  mice (6 ng/ml) (Table 1). Although levels of IL-6  
generated at this time by the ATP-challenged knockout animals were lower than those  
35 generated by their wild-type counterparts, they were elevated above those generated in  
response to PBS challenge (6 ng/ml when challenged with ATP versus 0 ng/ml when  
challenged with PBS, Table 1). This suggests that ATP affects IL-6 production via

5 both a P<sub>2x7</sub>R-dependent and -independent mechanism. Animals that were not primed with LPS yielded lavage fluids totally devoid of IL-1 and IL-6. Values in Table 1 represent the mean  $\pm$  standard deviation (N=6).

**Table 1**

Time Post Challenge (min)	PBS Challenge				ATP Challenge			
	IL-1 (ng/ml)		IL-6 (ng/ml)		IL-1 (ng/ml)		IL-6 (ng/ml)	
	WT	P <sub>2x7</sub> R <sup>-/-</sup>	WT	P <sub>2x7</sub> R <sup>-/-</sup>	WT	P <sub>2x7</sub> R <sup>-/-</sup>	WT	P <sub>2x7</sub> R <sup>-/-</sup>
30	0.05	0.04	5.6 $\pm$ 0.7	4.7 = 0.6	0.4 $\pm$ 0.12	0.04	8.2 $\pm$ 1.7	9 $\pm$ 1
120	0.02	0.02	1 $\pm$ 0.6	0	0.33 $\pm$ 0.08	0.02	16.8 $\pm$ 2.3	5.7 = 1.2

10

### EQUIVALENTS

15 The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.